

**A PHOSPHOENOLPYRUVATE SYNTHASE GENE USED IN PLANT PROTECTION**Field of the Invention

[0001] The present invention relates to a novel pathogenicity gene of plant bacterial pathogen. This gene encodes phosphoenolpyruvate synthase (*ppsA*) which is the key enzyme in gluconeogenesis. The gene and its product can be used as a novel target for plant disease control.

Description of the Prior Art

[0002] Plant disease is one of the most destructive factors lead to poor quality and low yield of crops. As the pathogen resistance to antibiotics and pesticides increases, the amount of pesticides is increasingly used in agricultural practices, which result in heavily environmental pollution and pesticide residue in crop production, and also raise agricultural costs. The environment friendly strategies and chemicals are pressing needed to be developed. The key to the development of novel chemicals and measurements in disease control is to understand the genetic mechanism of pathogenesis of plant pathogens, and genomics study provides novel approaches to explore the functions of the genes and the mechanisms of the interactions between plants and the pathogens on molecular level. Along with the development of DNA sequencing technology, the hot spots of the genomics are shifted from structural genomics, the main tasks of which are DNA sequencing, into the functional genomics where the identification of the functions of ORF and gene is becoming increasingly dominant. The high-throughput and large-scale works on the identification of gene function has laid a fundamental basis for newly developed biotechnology based industry. To date, pathogenic genes which have been identified include hypersensitive reaction and pathogenicity gene (*hrp*) (He 1998), avirulence genes (*avr*) (Bonas 1999), regulatory pathogenicity factors (*rpf*) (Barber 1997), gene involved in biosynthesis of exopolysaccharides (Tang, *et al.* (1991); Dow, *et al.* (2000a)), gene involved in biosynthesis of lipopolysaccharides (Dow, *et al.* (2000b)), etc.

[0003] Determining of the function of proteins predicted in the genome sequence is the major goal for the post-genome era. One of the direct ways for functional assignments is disrupting the gene and examining the phenotype of the mutant. The genome-wide mutagenesis

and mutant collection would provide important resources for the researches in bio-technology and life science on the genome level.

[0004] *Xanthomonas campestris* pathovar *campestris* (*Xcc*), a yellow pigmented  $\gamma$ -proteobacterium, is the causal agent of black-rot disease of crucifers, one of the most destructive diseases of cruciferous plant worldwide (Hayward, 1993). The present invention was based on the construction of mutant library of *Xcc* 8004 and genome-wide identification of novel pathogenicity genes and intended to provide the pathogenicity genes and their products to be used as a novel target for plant disease control.

#### Detailed Description of the Invention

[0005] It is an object of the present invention to provide a novel gene related to *Xanthomonas campestris* associated diseases.

[0006] The present invention provided the objective gene, phosphoenolpyruvate synthase gene, which has one of the following nucleotide sequences:

1. the nucleotide sequences of SEQ ID NO:1; and
2. a DNA sequence which has more than 80% homology with SEQ ID NO:1 and encodes a protein having the same function as the phosphoenolpyruvate synthase encoded by the sequence of SEQ ID NO:1.

[0007] The present invention provided a phosphoenolpyruvate synthase gene. Preferably, the gene has the DNA sequence of SEQ ID NO:1.

[0008] Plasmid pXC1950 harboring the phosphoenolpyruvate synthase gene was deposited in China General Microbiological Culture Collection Center on 27<sup>th</sup> Nov. 2003, with the accession number of CGMCC No. 1054, the identification reference of JM109/pXC1950, and the taxonomic designation of *Escherichia coli*.

[0009] The DNA sequence of SEQ ID NO:1 is part of the genomic DNA of *Xcc* 8004 strain. SEQ ID NO:1 consists of 2629 nts and contains the entire phosphoenolpyruvate synthase gene. The open reading frame (ORF) of the gene starts from 201nt from 5'-end to 2576 nt; the start codon TTG of the gene starts from 201nt from 5'-end to 203 nt; the stop codon TGA of the gene starts from 2577nt from 5'-end to 2579 nt, and the promoter region of the gene starts from 120 nt from 5'-end to 170 nt.

[0010] In the present invention, DNA sequences with sequence similarity of greater than 85%, 90%, 95%, 98% or 99% of SEQ ID NO:1 and encoding proteins having the same function of the phosphoenolpyruvate synthase encoded by SEQ ID NO:1, are preferred.

[0011] The amino acid sequence of SEQ ID NO:2 represents the deduced amino acid sequence of the phosphoenolpyruvate synthase gene, which consists of 792 amino acid residuals, with a molecular weight of 86.2 KD and an isoelectric point of 5.12.

[0012] The deduced phosphoenolpyruvate synthase consists of 792 amino acid residuals, and contains the pyruvate binding domain and the PEP-utilizing enzyme mobile domain.

[0013] The expression vectors containing the DNA sequences mentioned above are also intended to be included in the present invention.

[0014] The use of *XC1950* gene: Phosphoenolpyruvate synthase is the key enzyme in gluconeogenesis in bacterial pathogen. The inventors have discovered that gluconeogenesis is very important to the bacterial adaptation to adverse effects. Previous study has shown that the disruption of gluconeogenic pathway resulted in significant reductions in *Xcc* 8004 virulence. Inventors also have discovered the mutation of the phosphoenolpyruvate synthase will block the gluconeogenic pathway. That is to say, one can control the toxicity of bacteria if he/she can control the activity of phosphoenolpyruvate synthase gene or its product. Therefore, phosphoenolpyruvate synthase gene and its product can be used as targets in medicament treatment and plant disease control.

#### Description of the Drawings

[0015] Figure 1 shows the electrophoresis result of digested *XC1950* gene clone.

[0016] Lane 1:  $\lambda$ /*Hind*III DNA markers (DNA markers, from the largest to the smallest: 23.1kb, 9.4kb, 6.6kb, 2.4kb, and 2.0kb). Lane 2: The fragments of *XC1950* gene. Lane 3: The constructed plasmid pXC1950 digested with *Bam*HI+*Hind*III.

[0017] Figure 2 shows the electrophoresis result of the PCR result of *XC1950* deletion mutant.

[0018] Lane 1: 100bp DNA marker (DNA markers, from the largest to the smallest: 3kb, 2kb, 1.5kb, 1.2kb, 1kb, 0.9kb, 0.8kb, and 0.7kb). Lane 2: Total DNA from wild type *Xcc* 8004 strain as the templates. Lane 3-6: Total DNA from *XC1950* deletion mutants as the templates.

[0019] Figure 3 shows the growth pattern of *XC1950* mutants on medium which has pyruvate as its sole carbon source.

[0020] Colonies 1-4: The Tn5 insertion mutants of *XC1950* gene. Colonies 5-6: The deletion mutants of *XC1950* gene. Colonies 7-8: The complimentary strains of deletion mutants of *XC1950* gene. Colonies 9-10: Wild type *Xcc* 8004 strain.

[0021] Figure 4 shows the result of pathogenicity test of *XC1950* deletion mutants.

[0022] A. Wild type *Xcc* 8004 strain. B. The deletion mutants of *XC1950* gene. C. Water (used as negative control)

[0023] The following examples are offered to illustrate but not to limit the invention.

### Examples

#### Materials used in examples:

[0024] *Escherichia coli* JM109 strain and plasmid pGEM-3Zf(+) were purchased from Promega, Shanghai. Restriction enzymes and modification enzymes were purchased from Promega, Stratagene, and QIAGEN companies.

[0025] The host plant used in the example is Chinese radish (*Raphanus sativus* L. var. radiculatus Pers.), which was purchased from Sichuan Seed Company, China.

[0026] Wild type *Xcc* 8004 (See, for example, Tang JL et al. 1990. Cloning of genes involved in negative regulation of production of extracellular enzymes and polysaccharide of *Xanthomonas campestris* pathovar *campestris*. Mol Gen Genet. 222:157-160.); The Cosmid pLAFR1 and pLAFR3 (See, for example, Liu YN et al. 1990. A multipurpose broad host range cloning vector and its use to characterize an extracellular protease gene of *Xanthomonas campestris* pathovar *campestris*. Mol Gen Genet. 220:433-440.); Cosmid pPH1JI (See, for example, Hirsch PR et al. 1984. A physical map of pPH1JI and pJB4JI. Plasmid. 12:139-141.); Transposon Tn5gusA5 (See, for example, Zha D et al. 1998. Cloning of DNA sequences involved in exopolysaccharide synthesis of *Xanthomonas campestris* pv. *campestris*. Wei Sheng Wu Xue Bao. 38:251-255.).

[0027] Nutrient yeast extracts and glycerol (NYG) medium, containing 5.0 g/L polypeptone, 5.0 g/L yeast extracts, and 20.0 g/L glycerol, pH7.0.

[0028] Primers for gene *XC1950* amplification:

Up stream primer: XC1950-F: GGGGATCC TTTCAGCGGTGATACCGG

Down stream primer: XC1950-R: GGAAGCTT TGCGGCGGCCGCTCCCCG

[0029] The italicized letters indicate the additional restriction sites (*Bam*HI, *Hind* III); the squared letters indicate the protection nucleotides for restriction sites.

### Example 1

#### Construction of mutant library of *Xcc*

[0030] Cosmid pLAFR1 which can replicate in both *E. coli* cell and *Xcc* cell was used as the vector (pLAFR1::Tn5*gusA5*) to introduce the transposon Tn5*gusA5* into the wild type *Xcc* 8004 strain. The bacterial strain thus created was named as *Xcc* 8004/ pLAFR1::Tn5*gusA5*. Incompatible plasmid pPH1JI was then introduced into *Xcc* 8004/ pLAFR1::Tn5*gusA5* to drive out the pLAFR1::Tn5*gusA5*. Insertional mutants *Xcc* :: Tn5*gusA5* was screened on NYGA medium containing antibiotics (Kanamycin). The obtained DNA sequences were performed sequence alignment to the whole genome sequence of *Xcc* 8004 and the insertional site of each Tn5*gusA5* in the chromosome of the mutant was determined by TAIL-PCR (Thermal Asymmetric Interlaced-PCR) technique (Liu et al. 1995). Totally, 420 mutants with defects associated with pathogenicity, extracellular enzymes and polysaccharides were collected, 51 genes involved. Of the 51 genes, five were newly identified pathogenicity genes. The present invention relates only to *XC1950*, one of the five genes.

[0031] The deduced product of *XC1950* was predicted as phosphoenolpyruvate synthase and the BLASTP (a web-based bioinformatics tools developed by National Center for Biotechnology Information of USA, available at <http://www.ncbi.nlm.nih.gov/BLAST/>) result showed that *XC1950* contains phosphoenolpyruvate synthase/pyruvate phosphate dikinase domain at its N-terminal and PEP-utilizing enzyme domain at its C-terminal, indicating *XC1950* is phosphoenolpyruvate synthase gene and is involved in gluconeogenesis.

### Example 2

#### The cloning of *XC1950* gene (phosphoenolpyruvate synthase gene)

[0032] Primers (*XC1950-F* and *XC1950-R*) were designed according to the DNA sequence of *XC1950* gene. The intact DNA sequence of *XC1950* gene was amplified by PCR, using *XC1950-F* and *XC1950-R* as primers and the total genomic DNA of wild type *Xcc* 8004 as template (Fig. 1). The PCR product was cloned into pLAFR3, and the recombined plasmid was designated as pXC1950. The fragment of *XC1950* gene was subcloned from pXC1950 into pGEM3Zf(+) for long-term storage.

### Example 3

#### The construction and confirmation of the deletion mutant of *XC1950* gene

[0033] The Kanamycin gene (*Kan*) was amplified by PCR and cloned into vector pGEM3Zf(+). The up-stream and down-stream flanking sequences of *XC1950*, designated as *1950L* and *1950R*, were obtained by PCR. *1950L* and *1950R* were recombined to both ends of the cloned *Kan* fragment in pGEM3Zf(+), producing recombinant plasmid pGK1950 in which the *1950L*, *Kan* fragment, and *1950R* were tandemly ligated. Then, the fragment *1950L-Kan* fragment-*1950R* was subcloned into pLAFR3, producing pLGK1950. The pLGK1950 was introduced into wild type *Xcc* 8004 strain and the *Kan* fragment was replaced by *XC1950* gene via homologous cross-over at both *1950L* and *1950R* positions. After the introduction of incompatible plasmid pPH1JI by bi-parental mating, the conjugants were screen through culturing on NYGA medium containing antibiotics. The positive conjugants were confirmed by specific PCR. As shown in figure 2, the deletion mutants and the wild type *Xcc* 8004 produced significantly different product band patterns.

### Example 4

#### The growth pattern of the mutants of *XC1950* gene on culture medium with pyruvate as the sole carbon source.

[0034] On solid medium with pyruvate as the sole carbon source (2.0 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1g/ L Pyruvate, 4.0 g/L K<sub>2</sub>HPO<sub>4</sub>, 6.0 g/L KH<sub>2</sub>PO<sub>4</sub>, and 0.2 g/L MgSO<sub>4</sub> · 7H<sub>2</sub> O, 15g/L Agar, pH7.0), wild type *Xcc* 8004 strain, mutants, and the complimentary strain (mutant carrying the pXC1950) were inoculated by tip-dipping using autoclaved toothpicks and cultured under 28°C for 72 hours. The results showed that the mutants could not grow normally, but the wild type strain and the complimentary strain grew normally on the culture medium (Fig. 3), demonstrating that the mutation in *XC1950* gene caused *Xcc* defective in pyruvate utilization.

### Example 5

#### The pathogenicity test of *XC1950* gene mutant

[0035] The host plant used in the example is Chinese radish (*Raphanus sativus* L. var. radiculatus Pers.) and the leaf-clipping was used as inoculating method. The deletion mutants and the wild type strain were cultured in suspensions at 28°C for 15-18 hours, and were diluted

to OD600=0.2 before use. The mutants and the strains were inoculated into the radish leaves by cutting along the mid-vein of the leaf from a distance of 1-2 cm from the leaf tip with clippers which have been dipped in the suspensions for 5 seconds. The inoculated plants were kept in the warm and moisture greenroom at 25-30°C for 7 days. The wild type strain was used as positive control, and the water as the negative control. The result of plant test showed that the pathogenicity of mutants decreased significantly compared with the wild type and the complimentary strain, which indicated that the *XC1950* gene was directly involved in the pathogenicity of *Xcc*.

### References

- [0036] Barber CE et al. 1997. A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. Mol. Microbiol 24:555-566
- [0037] Bonas U et al. 1999. Gene-for-gene interactions: bacterial avirulence proteins specify plant disease resistance. Curr. Opin in Microbiology 2:94~98
- [0038] Dow M et al. 2000a. *Xylella* genomics and bacterial pathogenicity to plants. Yeast 17:263~271.
- [0039] Dow M et al. 2000b. The induction and modulation of plant defense resistances by bacterial lipopolysaccharides. Annu Rev Phytopathol 38:241-261.
- [0040] Hayward AC. 1993. The hosts of *Xanthomonas*. In: Swings G, Civerolo EL, editors. *Xanthomonas*. London: Chapman and Hall, pp. 1-119.
- [0041] He SY. 1998. Type III protein secretion systems in plant and animal pathogenic bacteria. Annu. Rev. Phytopathol. 36:363~392
- [0042] Hirsch PR et al. 1984. A physical map of pPH1JI and pJB4JI. Plasmid. 12:139-141.
- [0043] Liu YG et al. 1995 Efficient isolation and mapping of Arabidopsis thaliana T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J. 8:457-63.
- [0044] Liu YN et al. 1990. A multipurpose broad host range cloning vector and its use to characterise an extracellular protease gene of *Xanthomonas campestris* pathovar *campestris*. Mol Gen Genet. 220:433-440.
- [0045] Tang JL et al. 1991. Genetic and molecular analysis of a cluster of *rpf* genes involved in positive regulation of synthesis of extracellular enzymes and polysaccharide in *Xanthomonas campestris* pathovar *campestris*. Mol Gen Genet 226:409-417.

**[0046]** Zha D et al. 1996. Mutagenesis of *Xanthomonas campestris* pv. *campestris* with transposon Tn5gusA5 and isolation of mutants impaired with pathogenicity. Journal of Guangxi Agricultural and Biological Science. 15:23-29.

**[0047]** Zha D et al. 1998. Cloning of DNA sequences involved in exopolysaccharide synthesis of *Xanthomonas campestris* pv. *campestris*. Wei Sheng Wu Xue Bao. 38:251-255.